Effect of sperm DNA fragmentation on the clinical outcomes for in vitro fertilization and intracytoplasmic sperm injection in women with different ovarian reserves

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Objective: To investigate effect of sperm DNA fragmentation (SDF) on clinical outcomes of assisted reproductive technology in women with normal ovarian reserve (NOR) versus reduced ovarian reserve (ROR).
Design: Retrospective clinical study.
Setting: University-affiliated tertiary teaching hospital.
Patient(s): A total of 2,865 consecutive couples undergoing their first in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) cycle.
Intervention(s): SDF assessed using sperm chromatin dispersion in sperm samples 1–2 months before treatment.
Main Outcome Measure(s): SDF, IVF, and ICSI outcomes.
Result(s): The grouping criteria were [1] basal follicle stimulating hormone >10 IU/L, [2] antral follicle count <6, and [3] female age ≥38 years. Women fulfilling two of the three criteria were considered to have ROR, and those not meeting any criteria were considered to have NOR. The area under the receiver operating characteristic curve was 0.594 (0.539–0.648) for the ROR group and 0.510 (0.491–0.530) for the NOR group. A cutoff value for SDF to predict the clinical pregnancy rate (CPR) in the ROR group was 27.3%. When the SDF exceeded 27.3%, the live-birth and implantation rates in the ROR group were statistically significantly decreased, but the clinical pregnancy, live-birth, and implantation rates were not affected in the NOR group. The risk of early abortion increased significantly in the NOR group when the SDF exceeded 27.3%.
Conclusion(s): Sperm DNA fragmentation has a greater impact on IVF and ICSI outcomes among women with ROR, so SDF testing may be of particular clinical significance for these couples. (Fertil Steril® 2015;103:910–6. ©2015 by American Society for Reproductive Medicine.)
Key Words: IVF-ICSI outcome, ovarian reserve, sperm DNA fragmentation

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Sperm DNA integrity is required for accurate transmission of paternal genetic information and has been increasingly recognized as a promising biomarker of male infertility. Couples with a high percentage of sperm DNA fragmentation (SDF) have lower levels of natural fertility (1, 2). Furthermore, when SDF is extensive artificial insemination is not effective (3).

Many studies have been performed to elucidate any correlation between SDF and clinical outcomes of

conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (3–12). Some researchers have suggested that there is a strong negative correlation between SDF and the rates of fertilization, embryo development, implantation, and pregnancy (5, 8, 10, 12–14). Others have found no association between SDF and fertilization rate or embryo development, but have suggested an association between SDF and pregnancy (3, 6) or pregnancy loss (7). Furthermore, some studies have found no adverse effects of SDF on any clinical outcomes in IVF and ICSI (4, 15). Several factors—such as differing methods for detecting SDF, lack of standardized methods, varying patient selection criteria, and limited study sample sizes—may account for the inconsistency among study results. A meta-analysis by Collins et al. (16) revealed a small but statistically significant association between SDF and IVF-ICSI results, but the association was not strong enough to provide a clinical indication for the routine use of SDF testing in an infertility evaluation of men. However, some subgroups of couples with specific clinical characteristics may merit SDF testing once a strong association between SDF and IVF-ICSI results has been demonstrated.

Although the sperm chromatin structure assay (SCSA) is the most commonly used method to examine SDF, other methods such as terminal deoxynucleotidyl transferase-mediated terminal uridine nick-end labeling (TUNEL), the Comet assay, and sperm chromatin dispersion (SCD) have also been introduced into clinical practice. The SCD test, which is the simplest and least expensive method, can be performed using bright-field microscopy. Results obtained from the SCD test correlate well with those from other DNA integrity tests, such as SCSA and TUNEL (17–19). A recent study by Zhang et al. (19) suggested both TUNEL and SCD methods are effective, but the SCD appears to be more sensitive in detecting DNA damage. Recently, reports have proposed that the SCD test is useful for predicting IVF outcomes such as embryo development, fertilization rate (12, 14), and pregnancy rate (20). On the other hand, Anifandis et al. (21) suggested there is no correlation between embryologic data or pregnancy rate and SDF measured by the SCD test.

We investigated the effect of SDF, as measured by the SCD test, on the clinical outcomes of IVF and ICSI cycles in women with different ovarian reserves. We determined whether SDF has a greater impact on IVF and ICSI outcomes in certain subgroups, such as women with reduced ovarian reserve (ROR).

MATERIALS AND METHODS

Study Population

The study was approved by the medical ethics committees of our hospital. Data from a cohort of 2,865 consecutive women aged 20.5–46.5 years who underwent their first IVF (n = 2,085) or ICSI (n = 780) cycle between April 2009 and December 2012 at our reproductive medicine center were respectively analyzed in this study. Female patients with uterine pathology, including uterine synechia, adenomyosis, myoma, or other uterine abnormalities, were excluded from the study. Male patients who received vitamins, carnitine, or Chinese medicine before IVF and ICSI were excluded as well.

Sperm Analysis and SCD Test

Routine semen analysis and SDF assessment using an SCD test were performed 1–2 months before the initial treatment in all 2,865 cycles. Samples were collected by masturbation after 2 to 5 days of abstinence. After liquefaction, the samples were examined for concentration and motility according to the World Health Organization (WHO) laboratory manual for the examination and processing of human semen (22). Sperm morphology was evaluated following Tygerberg's strict criteria (23).

Each SCD assay was performed using the Halosperm kit (INDAS Laboratories) with a slight modification. In brief, the sperm concentration was diluted to 5–10 million per milliliter. The total volume was split into 30-μL aliquots on agarose gel in tubes that were placed in a water bath at 90–100°C for 5 minutes to fuse. The tubes were then placed in a water bath at 37°C for temperature equilibration. Next, 18 μL of the semen sample was added to each tube and mixed with the 30 μL of fused agarose, then 18 μL of the semen-agarose mixture was pipetted onto a precoated slide and covered with an 18 × 18-mm coverslip. The slide was placed in a refrigerator at 4°C for 5 minutes to allow the agarose to produce a microgel embedded with sperm cells. The coverslip was gently removed, and the slide was immediately immersed in an acid solution for 7 minutes. Next, the slide was immersed in lysing solution for 25 minutes. After 5 minutes of washing in a tray with abundant distilled water, the slide was dehydrated in increasing concentrations of ethanol (70%, 90%, and 100%) for 2 minutes each, and was then air dried.

For bright-field microscopy, each slide was covered with a mixture of Wright's staining solution and phosphate buffer solution (1:1) for 5–10 minutes. The slide was briefly washed in running water for 10 seconds and allowed to dry. Strong staining is preferred to achieve easy visualization of the periphery of the dispersed DNA loop halos. A minimum of 400 spermatozoa for each patient was scored according to the patterns established by Fernandez et al. (17). Sperm nuclei with fragmented DNA produce very small or no halos of dispersed DNA, whereas nuclei without DNA fragmentation release their DNA loops to form large halos. The percentage of sperm with very small or no halos was defined as the SDF level.

IVF and ICSI Procedures

The standard luteal long down-regulation protocol (LP) or the short flare-up protocol (SP) (24) was used in all treatment cycles. Briefly, with the LP a 0.5–0.9 mg depot of gonadotropin-releasing hormone (GnRH) agonist (Diphereline, 3.75 mg; Ipsen Pharma Biotech) was given in the midluteal phase of the preceding menstrual cycle. Stimulation with gonadotropin (Gonal-F; EMD Serono) was begun after 14 days (usually on cycle day 3–9) from the injection of Diphereline when pituitary down-regulation was achieved. Down-regulation was verified by an endometrial lining of 5 mm or thinner, a serum estradiol <50 pg/L, and a serum luteinizing hormone (LH) level <5 U/L. When SP was used, the GnRH agonist (Decapetyl, 0.1 mg; Ferring GmbH) was administered as a daily dose of 0.1 mg beginning cycle day 2 followed by gonadotropin (Gonal-F; EMD Serono) beginning on day 3. For both protocols, daily doses of 150 U or 225 U Gonal-F

were given for the first 5–6 days, and then adjusted according to follicle growth and the serum estradiol level. Human chorionic gonadotropin (hCG) 5,000–10,000 IU was administered when at least three follicles had reached 17 mm in diameter, and oocyte retrieval was performed 34–36 hours later.

The strategy for choosing the stimulation regimen was that LP was used in women with normal ovarian reserve (NOR) and SP in those with ROR. We used SP in women who fulfilled one of the three following criteria: [1] basal follicle-stimulating hormone (FSH) level of >10 IU/L, [2] antral follicle count (AFC) of <6, and/or [3] age ≥38 years. Otherwise, LP was used. Therefore, women treated with LP were considered to have NOR. In this study, women who met at least two of the three criteria above were considered to have ROR and were included in the ROR group.

All IVF and ICSI procedures were performed according to standard protocols (24). We used ICSI when the sperm concentration was less than 5×10^6/mL or when the motility was <10%. Each embryo transfer was performed on day 3 after oocyte retrieval. Embryo quality was assessed on day 2 after fertilization using the grading system described by Simon et al. (9). The embryo cumulative score was calculated by multiplying the embryo grade (A = 4, B = 3, C = 2, D = 1, and E = 0) by the number of blastomeres for each embryo. When a patient had more than one embryo, a mean of all embryos was calculated to obtain the total quality of all day-2 embryos. Clinical pregnancy was defined as the presence of a uterine fetal heart beat confirmed by ultrasound 4 to 5 weeks after embryo transfer. Early abortion was defined as spontaneous abortion earlier than 20 weeks of gestation.

Statistical Analysis

Continuous variables were presented as mean and standard deviation (SD), with independent *t* test or Mann-Whitney *U* test used for comparisons between pregnant and nonpregnant groups according to the normality of the data. The Kolmogorov-Smirnov test was performed to detect the normality of the data.

A receiver operating characteristic (ROC) curve analysis was performed to determine the prognostic accuracy of SDF as well as the cutoff point for predicting pregnancy in the NOR and ROR groups. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated for the cutoff points.

Univariate and multivariate logistic regressions were performed to determine the factors affecting clinical outcomes, including the clinical pregnancy rate (CPR), live-birth rate (LBR), and early abortion rate. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated to present the effect of the threshold SDF value. Univariate and multivariate linear regressions were performed to determine the factors affecting the implantation rate (IR), with β values and 95% CIs calculated to present the effect of the threshold SDF value. The variables included female age, retrieved oocytes, fertilization rate, cleavage rate, day-2 embryo quality score, number of embryos transferred, and the treatment type (IVF or ICSI). Confounders were the variables found to be statistically significantly correlated with clinical outcomes in univariate models. The adjusted OR or adjusted β with 95% CI were presented after adjustment for the confounders by the multivariate logistic or linear regression model. $P<.05$ was considered statistically significant. Data analyses were performed with SPSS 17.0 (SPSS, Inc.), and ROC analyses were performed with MedCalc statistical software (ver. 11.4.2.0; MedCalc Software).

RESULTS

Among the 2,865 consecutive cycles studied, the mean age of women in the ROR group (n = 327) was statistically significantly higher than that in the NOR group (n = 2,538) (36.1 ± 4.4 vs. 30.4 ± 3.5 years, respectively; $P<.01$). Statistically significantly fewer oocytes were retrieved in the ROR and NOR groups (3.9 ± 2.1 vs. 11.1 ± 5.0, respectively; $P<.01$). The CPR in the NOR group was 55.2%, and in the ROR group was 22.0% ($P<.01$). There was no difference in CPR between the 2,085 IVF cycles and 780 ICSI cycles (51.4% vs. 51.7%, respectively; $P>.05$).

The characteristics of the ROR and NOR groups categorized as pregnant and nonpregnant cycles are shown in Table 1. There were statistically significant differences between pregnant and nonpregnant cycles in the NOR group with respect to female age, male age, number of retrieved

TABLE 1

Baseline characteristics of the study population and clinical outcomes.

	NOR			ROR		
Characteristic	**Pregnant**	**Nonpregnant**	***P* value**	**Pregnant**	**Nonpregnant**	***P* value**
Transfer cycles (n)	1,401	1,137		72	255	
SDF (%)	18.4 ± 14.4	18.3 ± 13.5	.195	14.8 ± 6.9	21.0 ± 15.9	<.001
Female age (y)	30.2 ± 3.4	30.7 ± 3.5	<.001	35.5 ± 3.4	36.7 ± 4.7	.061
Male age (y)	32.7 ± 4.2	33.1 ± 4.3	<.01	37.9 ± 5.3	38.2 ± 5.2	.660
Retrieved oocytes (n)	11.3 ± 5.0	10.8 ± 5.0	<.01	4.3 ± 2.3	4.0 ± 2.4	.302
Fertilization rate (%)	74.2 ± 18.3	70.4 ± 20.8	<.001	82.8 ± 19.4	75.2 ± 24.9	<.01
Cleavage rate (%)	96.8 ± 7.9	94.7 ± 12.5	<.001	97.4 ± 7.8	95.8 ± 14.2	.204
Day-2 embryo quality score	8.6 ± 3.6	7.5 ± 3.6	<.001	9.3 ± 3.6	8.4 ± 4.4	.099
Embryos transferred (n)	2.2 ± 0.4	2.1 ± 0.5	<.001	2.3 ± 0.7	2.0 ± 0.8	<.001

Note: NOR = normal ovarian reserve; ROR = reduced ovarian reserve; SDF = sperm DNA fragmentation.

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oocytes, fertilization rate, cleavage rate, day-2 embryo quality scores, and number of embryos transferred. In the ROR group, there were statistically significant differences of SDF and number of embryos transferred between pregnant and nonpregnant cycles.

Effect of SDF on the Clinical Outcomes of IVF and ICSI in the ROR Group

According to the ROC curve analyses (Fig. 1A; Table 2), the SDF was statistically significant as a prognostic predictor of clinical pregnancy in the ROR group, with the area under the ROC curve (AUC) 0.594 (95% CI, 0.539–0.648; $P=.0066$). The value with the best ratio of sensitivity and specificity was 27.3%, which was used as the cutoff value (see Table 2). As shown in Table 3, the adjusted OR (95% CI) for CPR and LBR, and adjusted β (95% CI) for IR were 0.16 (0.05–0.54; $P<.05$), 0.07 (0.01–0.49; $P<.05$), and −11.30 (−18.50 to −4.10; $P<.05$), respectively, all of which were statistically significant when the SDF exceeded 27.3% in the ROR group.

Effect of SDF on the Clinical Outcomes of IVF and ICSI in the NOR Group

In the NOR group, the AUC was 0.510 (95% CI, 0.491–0.530; $P>.05$) (see Fig. 1B; Table 2), and no optimum cutoff threshold value could be obtained. When the SDF was >27.3%, the adjusted OR (95% CI) for CPR and LBR, and adjusted β (95% CI) for IR were not statistically significant (see Table 3). These results indicate that SDF was not an optimum prognostic predictor of CPR, LBR, or IR in the NOR group. However, the adjusted OR of 1.56 (95% CI, 1.05–2.32; $P<.05$) for the early abortion rate was statistically significant, suggesting that a high SDF significantly increased the risk of early abortion in the NOR group.

DISCUSSION

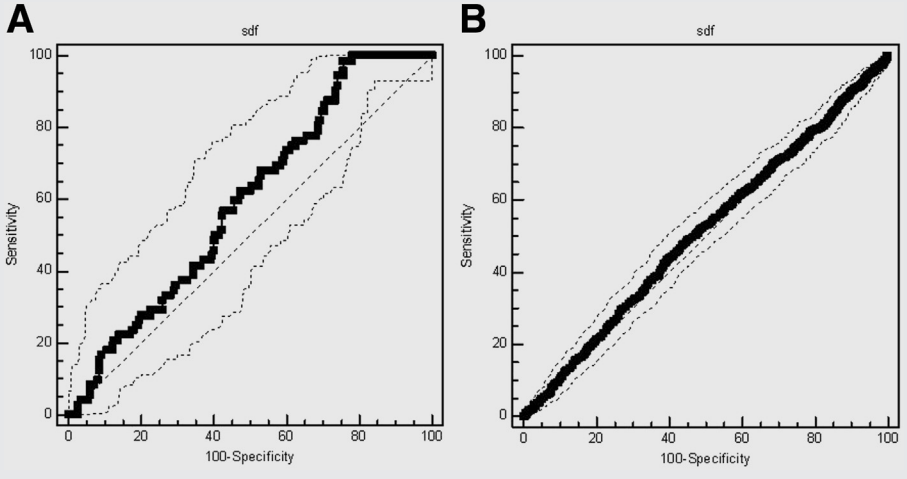
Many studies have been conducted to investigate whether SDF has a negative effect on IVF and ICSI outcomes (25). Some early studies did not clearly describe the ovarian status of the patients (4, 5, 13, 26, 27), and other studies included only female patients with NOR (6–9) or studied egg donors (11, 15, 28). Indeed, the pooling of women with different ovarian reserves could represent a major confounding factor in the analysis of the effect of SDF on IVF and ICSI outcomes. Furthermore, the effect of SDF on IVF and ICSI outcomes in women with ROR has not been reported previously. We investigated the effect of SDF level on IVF and ICSI clinical outcomes in women with ROR compared with those with NOR.

In our study, women with a high basal FSH level, older age, or decreased AFC were treated with the SP, whereas those considered to have NOR were treated with LP (29, 30). Women meeting at least two of the three indications (and thus with the greatest probability of having ROR) were categorized into the ROR group. The group's ROR status was confirmed by their having significantly fewer oocytes retrieved and an older mean age compared with the NOR group.

A ROC curve analyses found that the best AUC was statistically significant for the ROR group, but was not statistically significant for the NOR group. When SDF was >27.3%, the CPR, LBR, and IR were statistically significantly decreased in the ROR group but not in the NOR group. These findings clearly indicate a negative effect of SDF on CPR, LBR, and IR after IVF and ICSI in women with ROR that is not present in women with NOR.

Reduced ovarian reserve, a poor response to ovarian hyperstimulation, has been suggested to be related to reduced oocyte quality (31–33). Using pregnancy loss as a marker of oocyte quality, several studies found that pregnancy loss is significantly increased in women with elevated FSH levels

FIGURE 1



(**A**) Receiver operating characteristic (ROC) curve for sperm DNA fragmentation (SDF) in the reduced ovarian reserve (ROR) group. Area under the ROC curve (AUC) 0.594 (95% CI, 0.539–0.648) ($P=.0066$). (**B**) ROC curve for SDF in the normal ovarian reserve (NOR) group. AUC 0.510 (95% CI, 0.491–0.530) ($P=.373$).

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TABLE 2

Prognostic accuracy of sperm DNA fragmentation to predict the clinical pregnancy rate.

	NOR			ROR		
Statistical characteristic	**Pregnant**	**Nonpregnant**	***P* value**	**Pregnant**	**Nonpregnant**	***P* value**
SDF (%)	18.4 ± 14.4	18.3 ± 13.5	.915	14.8 ± 6.9	21.0 ± 15.9	.001
Area under ROC curve, (95% CI)	0.510 (0.491–0.530)		.373	0.594 (0.539–0.648)		.0066
Cutoff (%)	–			27.3		
Sensitivity (%), (95% CI)	–			98.6 (92.5–100.0)		
Specificity (%), (95% CI)	–			24.3 (19.2–30.1)		
Positive predictive value (%)	55.11			31.54		
Negative predictive value (%)	41.66			68.18		

Note: CI = confidence interval; NOR = normal ovarian reserve; ROR = reduced ovarian reserve; SDF = sperm DNA fragmentation; ROC = receiver operating characteristic.

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and decreased AFC, and in older women (31–33). In addition, a clear association has been established between female aging and poor oocyte quality, mainly due to aneuploidy (34). Several studies have also suggested a relationship between ROR and Down syndrome (35–37), possibly due to aneuploidy of the oocyte. Reduced ovarian reserve may harm the quality of remaining oocytes via elevated FSH (30, 38), oxidative stress (39), or defective microcirculation or granulose cell function (40). Thus, in this study the oocytes retrieved from the ROR group, as a whole, may have been of lower quality compared with those from the NOR group.

The quality difference in oocytes retrieved from the ROR and NOR groups in this study may explain the differing effects of SDF on the IVF and ICSI results in the two groups. Oocytes have been shown to have the capability to repair damaged DNA of spermatozoa in murine models (41–43). Although there is no direct evidence of such a repair mechanism in human oocytes, some studies using oocyte-donor IVF have suggested that the effect of SDF level on IVF and ICSI pregnancy outcome depends on oocyte quality (15, 28). In a study using oocytes from infertile patients, Meseguer et al. (28) reported that increased SDF had a statistically significant negative impact on the likelihood of pregnancy in IVF and ICSI cycles, whereas SDF did not have a statistically significant effect on oocyte donor cycles (28). Thus, the likelihood of failure or incomplete repair of SDF in fertilized oocytes from the ROR group was likely much higher than in the NOR group. This would result in a greater compromise of developmental capability of the resultant embryos in the ROR group, resulting in a decreased IR, CPR, and LBR.

The results of the NOR group in this study are not consistent with those of several studies conducted on women with NOR (6, 7, 9). Frydman et al. (6) found that the IR and CPR of IVF in women with NOR were significantly decreased in a group with higher SDF levels. With IVF using healthy and high-quality donor oocytes, Nunez-Calonge et al. (11) found that SDF affected pregnancy results. Our results, however, are in accordance with those of two other studies, which reported no negative impact of SDF on clinical outcomes of IVF and ICSI using donor oocytes (15, 28).

In our retrospective study, the risk of early abortion increased significantly in the NOR group, but not in the ROR group, when SDF exceeded 27.3%. Most previous studies did not have a large enough sample size to achieve statistically significant results (7). In our study, the increased risk of early abortion reaching statistical significance was probably due to the large sample size of the NOR group. This result is in agreement with the meta-analysis of Zini et al. (44). The small sample size of the ROR group may be

TABLE 3

Multivariate analyses of the association of sperm DNA fragmentation and clinical outcomes.

Outcome	**Group**	**Adjusted OR (95% CI)**	**Adjusted β (95% CI)**	***P* Value**	**Confounders adjusted**
Clinical pregnancy	NOR	1.14 (0.91–1.42)	–	.356	Female age, retrieved oocytes, cleavage rate, day-embryo quality score, embryos transferred
	ROR	0.16 (0.05–0.54)	–	.002	Female age
Live birth	NOR	0.99 (0.80–1.25)	–	.605	Female age, retrieved oocytes, cleavage rate, day-embryo quality score, embryos transferred
	ROR	0.07 (0.01–0.49)	–	.008	Female age, embryos transferred
Early abortion	NOR	1.56 (1.05–2.32)	–	.007	None
	ROR	24.47 (0.80–750.48)	–	.069	None
Implantation (rate)	NOR	–	0.82 (−3.20 to 4.84)	.832	Female age, retrieved oocytes, cleavage rate, day-2 embryo quality score, embryos transferred
	ROR	–	−11.30 (−18.50 to −4.10)	.022	Female age

Note: Reference is sperm DNA fragmentation <27.3%. CI = confidence interval; NOR = normal ovarian reserve; OR = odds ratio; ROR = reduced ovarian reserve.

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the reason the early abortion rate did not reach statistical significance.

Some studies have reported that high levels of SDF influenced IVF results but did not impact ICSI outcomes (7, 45). To the contrary, some studies have reported that SDF affected the ICSI outcomes but not IVF outcomes (10, 13). In our study, multivariate regression analysis did not find that treatment method (IVF or ICSI) was an independent confounding factor with respect to the association of SDF and clinical outcomes. Bungum et al. (3) reported a significantly higher IR and CPR with ICSI compared with IVF for patients with higher SDF levels, and recommended that ICSI be used when SDF exceeds 30%. However, such a recommendation should be considered cautiously because the most likely reason for the lack of impact of SDF on ICSI results is better ovarian reserve in the ICSI cycle, rather than the insemination method used.

Several researchers have tried to determine a SDF threshold to predict clinical outcomes of IVF and ICSI using the SCSA, TUNEL, and Comet assays (6, 7, 9). The threshold for predicting failure to achieve pregnancy was 27.3% in the ROR group using the SCD method in this study, which is comparable to a threshold of 25.5% found in another study using the SCD (46). In that study, the mean age of the population was 36.16 ± 4.48 years, which is also comparable to the age of our ROR group. A reference value of SDF for the prediction of compromised clinical outcomes of IVF and ICSI may, at least, depend on oocyte quality and thus is variable in different subgroups of women. Nevertheless, our study has shown that SDF examination is especially useful in men whose partners have ROR.

A weakness of our study is that SDF was measured on neat semen samples 1 to 2 months before the initiation of IVF or ICSI. The SDF level may have changed over this time period in some patients, although several studies have shown that SDF level remains stable over a long period of time (10, 47). In most previous studies, the SDF level was tested on sperm collected the day of oocyte retrieval (4, 6–8). However, Virro et al. (48) examined SDF using SCSA on semen samples collected before egg retrieval and found that SDF was related to fertilization, blastocyst development, and ongoing pregnancy in IVF and ICSI cycles. Furthermore, Speyer et al. (10) used SCSA testing on neat semen samples collected before egg retrieval and found a decrease in IR after ICSI with sperm with high SDF levels. Nevertheless, our results and the results of these studies indicate that SDF tested using raw samples before initiation of IVF and ICSI treatment is helpful in predicting the clinical outcomes of IVF and ICSI.

In conclusion, the results of our study show that SDF has a negative impact on the clinical outcomes of IVF and ICSI, especially in women with ROR. Oocyte quality might be the pivotal determinant for the negative effect of SDF. Our findings suggest that SDF testing is especially useful for couples with ROR and high SDF levels seeking IVF and ICSI. Some studies have shown that certain medical treatments could be helpful in alleviating SDF (49, 50). However, further research is warranted to determine the usefulness of these treatments in improving the clinical outcomes of IVF and ICSI in couples with ROR and high SDF levels.

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